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JOURNAL OF SURGICAL RESEARCH 59, 299–304 (1995)

Hepatic Function Is Preserved Following Liver-Directed, Adenovirus-Mediated Gene Transfer[1]

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Submitted for publication November 30, 1993

Inherited and acquired disorders of the liver are attractive targets for gene therapy. Hepatic cells are susceptible targets for shuttle vectors because of a diversity of protein and viral receptors and accessibility of a selective afferent blood supply. Preservation of existing hepatic cell integrity and metabolic function is of paramount importance for successful whole animal gene therapy trials. In this report, we examine hepatic cell function and integrity following adenovirus-mediated reporter gene transfer to the liver *in vivo*. E1-deleted, replication-defective adenovectors encoding the LacZ gene driven by the human CMV promoter were delivered to the liver by isolated portal perfusion. The gene transfer rate, as determined by specific histochemical staining, approached 30% with recombinant protein detectable by Western blot throughout the course of study. Hepatic cell integrity as assessed by histology and hepatic enzyme profile (serum aspartate aminotransferase, γ-glutamyl transpeptidase) demonstrated normal cellular architecture and no significant difference between transfected liver and controls. Hepatic synthetic and metabolic function, as determined by albumin levels, prothrombin time, and bilirubin, were similar between the two study groups. This study demonstrates that efficient adenovirus-mediated gene transfer and expression in the rat liver do not compromise hepatic cell metabolism and integrity. © 1995 Academic Press, Inc.

INTRODUCTION

The principal goal of liver-directed gene therapy is the introduction of functional genes into hepatic cells to correct genetic deficiencies or alter a pathologic process. Current methods of recombinant gene delivery to liver cells utilize protein–DNA complexes [1] or replication-defective viral particles [2]. Compared to other vectors, replication-defective adenoviruses are highly attractive for hepatic gene therapy because (a) high purity concentrates of adenovirus vectors may allow high multiplicity of infection (m.o.i.) *in vivo*, (b) the shuttle vector can insert DNA sequences into hepatic cells regardless of the organ's physiologic state (i.e., quiescent or regenerating), and (c) specific serotypes are highly hepatotropic [3–4] and rapidly endocytosed by eukaryotic cells [5]. Few whole animal studies have described the use of adenoviral vectors for genetic lesions of the liver, although reports describing regional and systemic administration of adenoviral vectors have demonstrated hepatic expression of recombinant genes for variable duration [6–8]. These studies suggest wide variability of persistence and expression within targeted cells. Preservation of existing hepatic cell integrity and metabolic function is of paramount importance for successful whole animal gene therapy trials. However, it is still unclear whether adenoviral-mediated gene expression in levels sufficient for therapeutic efficacy is injurious to normal metabolic and synthetic function of the organ.

In the current study, we examined liver function and markers of cell injury following adenovirus-mediated gene transfer in a sham model of orthotopic liver transplantation. High-titer adenovectors encoding the LacZ gene were delivered selectively to the rat liver via isolated portal perfusion. Liver enzymes and histology were examined for evidence of hepatic injury, and standard assays for liver protein synthesis were measured in parallel to recombinant protein levels.

METHODS

Viral vectors. The adenoviral vector AdHCMVsp1-LacZ contains an expression cassette encoding the *Escherichia coli* β-galactosidase gene (LacZ) under control of the human immediate early CMV promoter upstream of a polyadenylation signal. Because the expression cassette replaces the entire viral E1a region and most of the

[1] This work was partially supported by NCI Training Grant CA09010-18 (K.E.D.), NIH-PO1 CA59326-01 (A.S.), and American Cancer Society Institutional Grant to the Jonsson Cancer Center/UCLA Medical Center, IN/IRG 131-L (A.S.).

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0022-4804/95 $12.00

E1b region (early viral proteins), this vector is replication-defective. The virus vectors are able to replicate only in 293 cells, a human embryonic kidney line which contains the Ad E1 region in chromosomal DNA and constitutively expresses the missing E1 proteins. Viral stocks are prepared by vector infection of 293 cells and yields are defined by plaque assays and expressed as plaque forming units (PFU). In the current study, a viral titer of 5×10^9 PFU/ml was used exclusively.

In situ liver perfusion. Male Lewis rats weighing 250–300 g were used. After induction of anesthesia, the abdomen was entered through a broad transverse incision. The right adrenal, coronary, and gastroduodenal veins were ligated. The liver was temporarily excluded from the circulation by clamping the portal vein, hepatic artery, and suprahepatic and infrahepatic vena cava. The liver was asanguinously perfused by portal vein cannulation at 2.5 ml/min with 25 ml at 4°C. Effluent was collected from a cannula in the infrahepatic vena cava. Perfusion time (cold ischemic time) was held constant at 10 min and vessels were repaired primarily, followed by reperfusion of the organ with blood.

Study groups and experimental design. Animals were grouped as follows: Group 1, negative controls ($n = 24$), were perfused with 25 ml of lactated Ringer's (LR); Group 2 livers ($n = 24$) were perfused with 5×10^9 PFU AdHCMVsp1LacZ in LR. To document gene transfer and expression, vector DNA, LacZ transcription, and recombinant β-galactosidase production were assayed from liver biopsies. Animals were sacrificed at 1, 3, 5, and 7 days after operation ($n = 6$ per day).

Liver function tests. To assess the integrity of hepatic synthetic function and the extent of cellular injury following gene transfer, standard liver enzyme profiles were measured from 1-ml aliquots of arterial blood prior to exsanguination. A clinical veterinary laboratory assayed for rat serum aspartate aminotransferase (SGOT), γ-glutamyl transpeptidase (GGT), prothrombin time (PT), albumin, and bilirubin.

Histology. Liver biopsies obtained over the specified intervals were rinsed in phosphate-buffered saline and then frozen in liquid nitrogen. Cryostat sections (8 μm) were cut onto glass slides. To determine infection rate, freshly mounted slides were immersed in 1.25% glutaraldehyde for 10 min, and then incubated for 4 hr at 37°C in X-gal staining solution: 5 m*M* $K_4Fe(CN)_6$, 5 m*M* $K_3Fe(CN)_6$, 2 m*M* $MgCl_2$, and 2 μg/ml of X-gal (5-bromo-4-chloro-indolyl-β-D-galactopyranoside, Boehringer Mannheim, Indianapolis, IN). Cells expressing β-galactosidase cleave colorless X-gal to yield a blue chromophore in the cytoplasm. Representative areas were scored for percentage of infected (blue-staining) cells at 10× and 40× magnification by an observer unaware of the experimental conditions. Slides were counterstained with hematoxylin and eosin or eosin alone; liver architecture and inflammation were assessed by an experienced pathologist.

DNA/RNA polymerase chain reaction. Successful gene transfer was confirmed by the presence of adenovirus sequences within infected liver using the polymerase chain reaction (PCR). Flanking primers were designed to detect a 535-base-pair (bp) DNA sequence from the adenovirus E4 region (primer 1, CGCAGGGCCAGCTGAAC; primer 2, GGCTTTATCGGGCCCC), downstream from the LacZ gene, within infected animal viscera. Total liver DNA was extracted from homogenized biopsies using phenolchloroform followed by ethanol precipitation. One microgram of DNA was amplified by PCR in a total volume of 50 μl with 0.2 *M* each of deoxynucleoside triphosphate, 0.25 m*M* of 5'- and 3'-oligonucleotide LacZ primers, 50 m*M* KCl, 5 m*M* Tris–Cl (pH 8.3), 1.5 m*M* $MgCl_2$, and 2.5 U *Taq* polymerase (Perkin–Elmer Cetus, Norwalk, CT). PCR was performed in a DNA thermal cycler (Perkin–Elmer) using the following amplification profile: 34 cycles of denaturing for 1 min at 90°C, primer annealing at 60°C for 2 min, and extension at 72°C for 2 min. PCR products were separated by 1.2% agarose gel electrophoresis and stained with ethidium bromide. Positive controls for these experiments were viral-producing 293 cells.

Total liver RNA was extracted by the one-step RNAzol method [9]. Five micrograms of extracted total RNA was incubated twice for 15 min at 37°C with 25 U RNase-free DNase I (Pharmacia) in 20 ml of 40 m*M* Tris–HCl (pH 7.5), and 6 m*M* $MgCl_2$. RNA was then extracted with chloroform and precipitated with ethanol in the presence of 100 ng dextran. One microgram of RNA was converted to cDNA using reverse transcriptase, then amplified by PCR, and electrophoresed as described above. The amplified DNA was transferred to nitrocellulose [10], hybridized with a 500-bp LacZ fragment labeled with [γ-^{32}P]-dCTP, and autoradiographed. Positive controls for these experiments were RNA extracts from virion-producing 293 cells. Oligomeric primers which flank an internal 1036-bp sequence of the LacZ gene were employed to demonstrate transcription of the transferred sequences (primer 1, GCCGACCGCACGCCGCATCCAGC; primer 2, CGCCGCGCCACTGGTGTGGGCC). E4 primers were used to detect viral message, indicative of replication-competent adenovirus.

Recombinant protein detection. Protein was extracted from homogenized liver biopsies using 0.01 *M* Tris, pH 7.6, 0.21 *M* NaCl, 0.1 *M* MgOAc, 0.1 *M* β-mercaptoethanol, and 0.001 *M* EDTA. Sample protein concentrations were determined by a variation of Lowry's method [11]. Aliquots (1.5 μg) of protein were then electrophoresed in 10% SDS–PAGE and transferred to nitrocellulose. After blocking for nonspecific binding with 10% dry milk, the membrane was incubated with a primary anti-E.coli-β-galactosidase monoclonal antibody

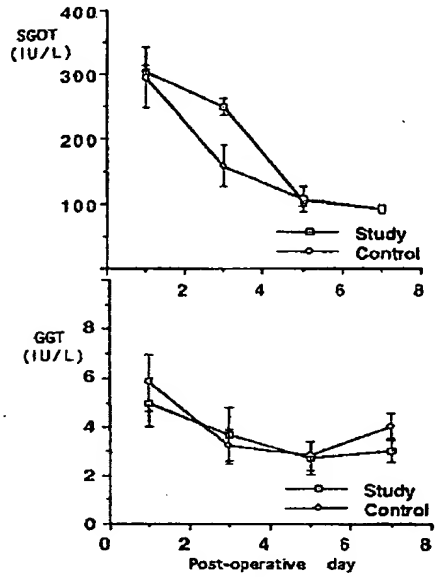

FIG. 1. Liver function tests revealed similar aspartate aminotransferase (SGOT) and γ-glutamyl transpeptidase (GGT) throughout the course of study in control and adenovirus-perfused animals. GGT levels were consistently within normal limits. SGOT levels were high initially (normal range 97–281 IU/liter), reflecting ischemic or reperfusion injury to the liver.

(mAb), washed, and incubated with a secondary mAb conjugated to horseradish peroxidase. Pure recombinant β-galactosidase (Sigma) was used as a positive control.

Statistical methods. Results of liver function tests were expressed as the mean ± standard error of the mean. Group comparisons were performed by Student's *t* test. Differences were considered significant at $P < 0.05$. Statistical analysis was performed using the StatView II program.

RESULTS

Liver function tests. Ischemic and preservation injuries after liver transplantation are well-correlated with serum aspartate aminotransferase levels, as well as elevation of total and nonconjugated bilirubin. In this study total and conjugated bilirubin were within normal range in control and transfected livers (0.2–0.4 mg/dl, $P = 0.46$) throughout the study period. In addition, there was no evidence of bile duct injury as reflected by GGT levels (Fig. 1). In contrast, SGOT values measured in Groups 1 and 2 were similarly elevated to twice normal at 24 hr following *in situ* perfusion, but returned to the normal range (97–281 IU/liter) by Postoperative Day 5 (Fig. 1). We postulate that this initial SGOT elevation may be reflective of surgical damage occurring during mobilization of the liver prior to perfusion, as well as ischemic injury during and immediately after reperfusion. To test whether such injury resulted in interference with the functional capacity of the liver, synthetic liver function was assayed. Prothrombin time in the transfected and control groups was slightly increased in the first 5 days after perfusion, but remained within the normal range throughout the course of study (21–25 sec, $P = 0.4$) (Fig. 2). Long-term survivors have also demonstrated normal coagulation parameters. Albumin levels were also normal between groups immediately after surgery and at each time point up to Day 7 (Fig. 2). A slightly lower albumin level was detected in Group 2 animals on Postoperative Day 7, but this difference was not statistically significant. These results also suggest that, despite efficient infection rate and recombinant protein synthesis (see below), native liver protein production was not affected.

Histology. At the time of sacrifice, transfected and control livers appeared grossly normal. No clinical findings of liver dysfunction were evident at the time of sacrifice, such as ascites, splanchnic venous congestion, or hepatic venous congestion. Liver biopsies stained with hematoxylin and eosin revealed intact liver architecture in infected animals, indistinguishable from normal controls. No cellular infiltrates were observed in infected or control livers throughout the course of study. There was no parenchymal cell ballooning or rounding characteristic of a lytic infection in AdHCMVsp1LacZ-infected or control sections.

X-gal staining revealed pockets of blue hepatocytes

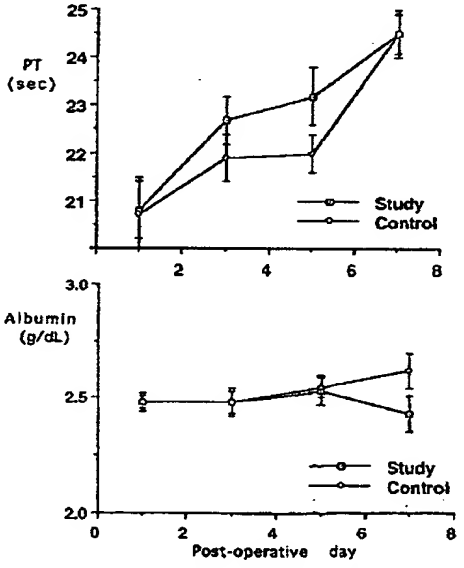

FIG. 2. Markers of hepatic synthetic function revealed normal prothrombin times (PT) in both study and adenovirus-perfused animals throughout the course of study. (Normal rat PT is 21–25 sec). Similarly, albumin levels were normal throughout in both groups (normal range 2.4–4.2 g/liter), with no difference between groups.

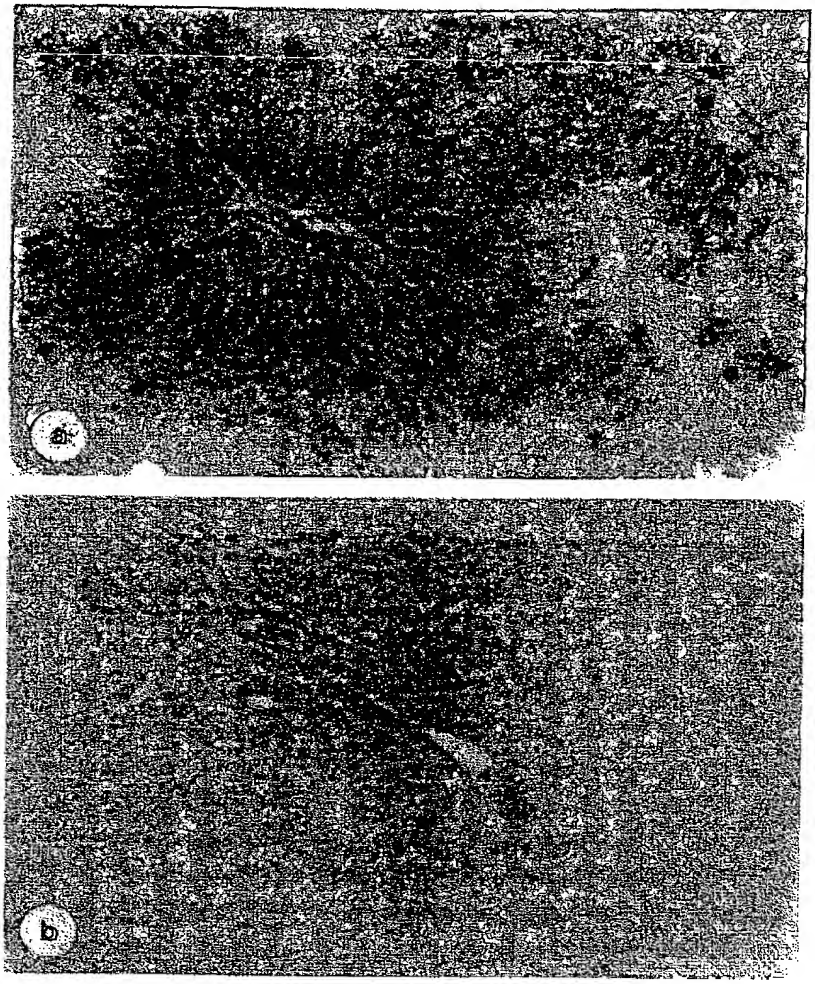

FIG. 3. Histologic sections of liver from rats sacrificed on Postoperative Day 1, stained with eosin and X-gal. Control livers did not stain with X-gal while AdHCMVsp1LacZ-perfused liver contained diffuse blue-staining, with 30% of hepatocytes demonstrating the recombinant protein (a). Staining was particularly concentrated in zones 1 and 2.

approaching 30% of cells under low (10×) and high (40×) magnification 24 hr after operation (Fig. 3a). Stained parenchymal cells, found only in AdHCMV-sp1LacZ-perfused livers, elicited a blue chromophore exclusively in the cytoplasm. Blue-stained liver cell cords were dispersed throughout the acinar inlet, predominantly in zones 1 and 2. In contrast, a relative scarcity of blue parenchymal cells was seen around the portal triads or hepatic veins. Liver biopsies of control (LR-perfused) animals did not stain blue with X-gal (Fig. 3b). The results demonstrate that adenovirus vectors delivered via portal vein predominantly transfect hepatocytes, with gene transfer concentrated at parenchymal cells within the centrilobular zones. Using these methods, it was impossible to determine infection rate of nonparenchymal cells. Future studies with *in situ* hybridization as well as FACS analysis will assist in identifying infected cell subpopulations.

DNA/RNA/protein analyses. Neither adenovirus nor β-galactosidase sequences were detected by DNA PCR in Group 1 samples (negative control). By contrast, positive control 293 cells and livers perfused with Ad-HCMVsp1LacZ manifested E4 sequences, confirming gene transfer by replication-defective adenovirus. The presence of E4 sequences up to 7 days after gene transfer correlated with X-gal staining. DNA from lung, spleen, and muscle in Group 2 animals did not contain detectable levels of adenoviral sequences, confirming specific localization of gene transfer to liver after isolated perfusion.

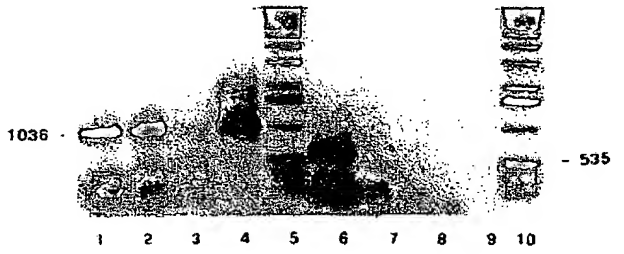

FIG. 4. RNA PCR revealed a characteristic band at 1036 bp for LacZ sequences in AdHCMVsp1LacZ-infected livers (POD 1-lane 1, POD 7-lane 2) and 293 cells (positive control, lane 4), but not saline perfused livers (lane 3). In contrast, adenoviral E4 sequences (535 bp), indicating viral RNA production, were present only in the vector-producing cells (293 cells, lane 6), but not in AdHCMVsp1LacZ-infected (POD 1-lane 7, POD 7-lane 8) or saline perfused livers (lane 9).

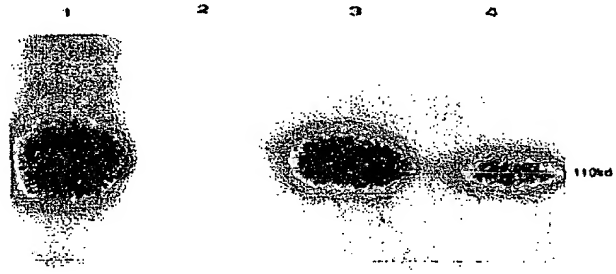

FIG. 6. Western blot from AdHCMVsp1LacZ-perfused livers demonstrated a 110-kDa (β-gal) band, comigrating with pure β-gal (lane 1). Lane 2, LR-perfused liver (negative control); lanes 3 and 4, adenoviral vector-perfused livers, POD 1 and 7.

Expression of both β-galactosidase and the E4 region was detected in RNA isolates from infected 293 cells, confirming transcription of both the β-galactosidase gene and the early viral proteins in 293 cells. Liver RNA from LR-perfused animals (Group 1) had no detectable levels of either adenoviral or LacZ RNA. Liver specimens from Group 2 had transcripts specific for β-galactosidase as early as 24 hr after gene transfer and up to 7 days postoperatively (Figs. 4–5). Liver perfused with AdHCMVsp1LacZ did not express E4 transcripts, verifying the absence of replication-competent, wild-type adenovirus expression (data not shown).

Recombinant protein production in AdHCMVsp1-LacZ-perfused liver was assessed by Western blotting. Protein extracts from AdHCMVsp1LacZ-infected liver (Group 2) contained a 110-kDa protein comigrating with purified recombinant *E. coli* β-galactosidase (Fig. 5). Negative controls (Group 1) expressed no β-gal.

CONCLUSION

The application of gene therapy to hereditary and acquired liver disorders in whole animal models is the focus of considerable attention. Replication-defective viruses are useful instruments for mediating gene transfer to organs *in vivo* because of their natural tropism for mammalian cells and because they can be rendered replication-defective and thus nonpathogenic. A rising interest in human adenoviruses as vectors for *in vivo* liver-directed gene therapy has developed for several reasons, including serotype tropism for hepatic cells, efficient infection of nondividing cells (i.e., hepatocytes), and relative stability and amenability to purification and concentration. These characteristics have permitted investigators to explore methodologic and therapeutic studies using adenoviral vectors for liver disease in whole animal models.

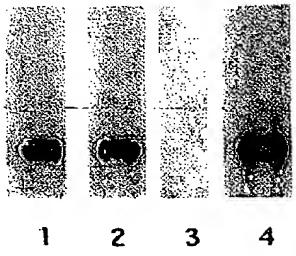

FIG. 5. Southern blot of reverse transcriptase PCR shows hybridization of [γ-^{32}P]dCTP-labeled LacZ probe to AdHCMVsp1LacZ-infected livers (POD 1-lane 1, POD 7-lane 2) and 293 cells (positive control, lane 4), but not saline perfused livers (lane 3).

The method of gene transfer described in this study, using portal perfusion, resulted in efficient targeting, with expression of recombinant protein in at least 30% of liver cells. A previous study describing bolus injection of 1×10^{10} adenovectors encoding human α-1 antitrypsin and β-galactosidase cDNA into rat portal vein resulted in a 1% infection rate [7]. In another study, however, when mice were given systemic administration of 5×10^8 adenovectors, functional expression of the inserted cDNA was localized to 90% of the liver cells and lasted for 15 months [8]. Interestingly, only in the murine model was an inflammatory infiltrate noted in liver after gene transfer.

The present study affords ample histologic evidence that hepatocytes are the primary targets of adenoviral vectors *in vivo*. A distinct hepatocyte population appears to be susceptible by portal vein delivery; that is, hepatocytes located around the acinar inlet. These cells are situated within geometrically tortuous sinusoids which favor solute uptake and cell contact [15]. We are not able to exclude that nonparenchymal hepatic endothelial cells are also infected and express the recombinant gene product at lower levels. Further studies will address this issue.

Liver injury by adenovirus-mediated gene transfer may be mediated by several mechanisms. Following adenovirus translocation into the cell cytoplasm by recep-

tor-mediated endocytosis, lysosomal fusion with the endosome-bound virus is disrupted by the viral penton fiber, thereby permitting adenovirus DNA entry into the nucleus [16]. This process might induce hepatocyte damage. In addition, excessive shunting of cell machinery to transcribe vector sequences may compromise housekeeping functions of the hepatocyte. The ability of adenovectors to direct high level expression is reflective of promoter strength and the number of viral copies per cell. The relatively strong promoter in our viral construct (human immediate early CMV) can direct up to 3% of cell protein production toward recombinant gene expression in tissue culture [17]. In the present study, however, serum enzyme markers characteristic of hepatic cell injury were not significantly elevated over control values. Hepatocellular synthetic function of the transfected livers was comparable to control samples. Metabolic indicators of liver function such as bilirubin conjugation and secretion were also unperturbed despite significant intracellular deposits of β-galactosidase. Our model used a m.o.i. of 5:1 (5×10^9 viral particles for approximately 1×10^9 hepatocytes). This relatively low m.o.i. probably accounts, in part, for the laboratory patterns of preserved hepatocellular function. At multiplicities of infection greater than 50:1, we have observed hepatic necrosis, ascites, and liver failure in a similar model [14].

In conclusion, replication-defective adenoviruses are capable of efficient genetic modulation of organs, such as the rat liver, without compromising normal cellular pathways, when used at appropriate multiplicities of infection. In animals, the precise levels of recombinant protein required for adequate therapeutic benefit have yet to be determined for any pathologic state. The adenovirus system may currently be ideally suited for *in situ* gene therapy, but further studies are required to assure efficacy and lack of pathogenicity in different species, and to determine whether prior exposure to adenoviruses affect transfection and/or damage of the targeted liver.

REFERENCES

1. Wu, G. Y., and Wu, C. H. Receptor-mediated gene delivery and expression *in vivo*. *J. Biol. Chem.* **263**: 4621, 1988.
2. Ledley, F. D., Darlington, G. J., Hahn, T., and Woo, S. L. C. Retroviral gene transfer into primary hepatocytes: Implications for genetic therapy of liver specific function. *Proc. Natl. Acad. Sci. USA* **84**: 5335, 1987.
3. Michaels, M. G., Green, M., Wald, E. R., and Starzl, T. E. Adenovirus infections in pediatric liver transplant recipients. *J. Infect. Dis.* **165**: 170–174, 1992.
4. Koneru, B., Atchison, R., Jaffe, R., Cassavilla, A., Van Thiel, D. H., and Starzl, T. E., Serological studies of adenoviral hepatitis following pediatric liver transplantation. *Transplant Proc.* **22**: 1547–1548, 1990.
5. Longberg-Holm, K., and Philipson, L. Early events of virus-cell interaction in an adenovirus system. *J. Virol.* **4**: 323–328, 1969.
6. Stratford-Perricaudet, L. D., Levrero, M., Chasse, J. F., Perricaudet, M., and Briand, P. Evaluation of the transfer and expression in mice of an enzyme-encoding gene using a human adenovirus vector. *Human Gene Ther.* **1**: 241–256, 1990.
7. Jaffe, H. A., Danel, C., Longeneker, G., Metzger, M., Setoguchi, Y., Rosenfeld, M. A., Gant, T. W., Thorgeirsson, S. S., Stratford-Perricaudet, L. D., Perricaudet, M., Pavirani, A., Lecocq, J. P., and Crystal, R. G., Adenovirus-mediated *in vivo* gene transfer and expression in normal rat liver. *Nature Genet.* **1**: 372–378, 1992.
8. Herz, J., and Gerard, R. D. Adenovirus-mediated transfer of low density lipoprotein receptor gene acutely accelerates cholesterol clearance in normal mice. *Proc. Natl. Acad. Sci. USA* **90**: 2812–2816, 1993.
9. Chomczynski, P. RNAzol B method. *Cinna-Biotex Bull.* **3**: 1, 1989.
10. Southern, E. M. Detection of specific DNA sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* **98**: 503–517, 1974.
11. Bradford, M. A. A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein dye binding. *Anal. Biochem.* **72**: 248–254, 1976.
12. Mulligan, R. C. The basic science of gene therapy. *Science* **260**: 926–932, 1993.
13. Bloom, H. H., Forsyth, B. R., Johnson, K. M., *et al.* Patterns of adenovirus infection in Marine Corps personnel. I. A 42 month survey in recruit and nonrecruit populations. *Am. J. Hygiene* **80**: 328–342, 1964.
14. Shaked, A., Csete, M. E., Drazan, K. E., Berk, A. J., and Busuttil, R. W. Adenovirus-mediated gene transfer into syngeneic rat liver grafts. *Transplantation*, in press.
15. Arias, I. M., Jakoby, W. B., Popper, H., Schachter, D., and Shafritz, D. A. (Eds.) *The Liver: Biology and Pathobiology.* Second ed. New York: Raven Press, 1988. Pp. 213–225.
16. Chardonnet, Y., and Dales, S. Early events in the interaction of adenoviruses with HeLa cells. I. Penetration of type 5 and intracellular release of the DNA genome. *Virology* **40**: 462–477, 1970.
17. Wilkinson, G. W. G., and Akrigg, A. Constitutive and enhanced expression from the CMV major IE promoter in a defective adenovirus vector. *Nucleic Acids Res.* **20**: 2233, 1992.